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13. ABSTRACT *(Maximum 200 Words)*
Systemic drugs are one of the most potent means for controlling beast cancer. Their ability to kill cancer cells often depends on the presence of appropriate physiological conditions, *e.g.*, the uptake and retention of 5-fluorouracil (5-FU) may be influenced by pH. This opens the possibility of predicting which tumors will show best response by measuring pH *a priori* or modulating tumor physiology to optimize tumor selectivity. We have developed a <u>novel class of non-invasive NMR pH indicators</u> and propose a novel second generation of enhanced indicators to investigate breast tumor pH.
We have now successfully synthesized our first trifluoromethyl pyridoxol pH indicator, which displays a narrow 19F NMR resonance and is sensitive to pH. This molecule has the advantage of three times the signal to noise of previous indicators. It has been tested in blood and will be evaluated in breast tumors in the near future. We will also investigate the feasibility of manipulating pH in order to enhance breast tumor uptake. An exciting additional finding is that the NMR approach is potentially suitable for assaying additional tumor characteristics and we have invented a novel concept in testing for gene transfection.

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Table of Contents

Introduction

Systemic drugs are one of the most potent means for controlling breast cancer. Their ability to kill cancer cells often depends, however, on the presence of appropriate physiological conditions. For instance, exciting recent results suggest that uptake and retention of 5-fluorouracil (5-FU) in tumors may be influenced by pH, in particular, the trans membrane pH gradient. This opens the possibility of predicting which tumors will show best response by measuring pH *a priori* or modulating tumor physiology to optimize tumor selectivity. We have developed a novel class of non-invasive NMR pH indicators and propose a novel second generation of enhanced indicators, which we will use to investigate breast tumor pH. Specifically, we will survey a series of diverse breast tumors in order to examine the specific correlation of 5-FU uptake versus pH gradient. In addition, we shall investigate the feasibility of manipulating pH in order to enhance breast tumor uptake.

We have three specific aims for this project:

Phase 1 Design, synthesis and evaluation of next generation molecules:
Based on our experience with 6-fluoropyridoxamine (6-FPAM) [1, 2], we have designed a second generation of enhanced pH indicator incorporating a trifluoromethyl group. These will provide three times greater signal intensity, improving the precision, and accuracy, of pH measurements *in vivo*. We will synthesize and characterize this new generation of improved ^{19}F NMR pH indicators.

Phase 2 Evaluation of a novel series of pH indicators *in vivo*:
Test trifluoromethyl pyridoxol in breast tumors *in vivo*. We believe the proposed pH indicators incorporating CF_3 reporter groups will offer considerable advantages over our current pH indicator 6-FPAM. However, should they fail to meet expectations (*e.g.*, due to toxicity or failure to cross cell membranes) then our current best reporter molecule (6-FPAM) will be used. These fluorine NMR pH indicators exhibit very strong NMR chemical shift response to changes in pH. They readily permeate cells providing simultaneous measurement of intra- and extracellular pH, and hence, the pH gradient.

Phase 3 Application of the new molecules to critical issues in breast cancer:
We will compare the regulation of pH gradients in two human and two rat tumor sublines that exhibit diverse therapeutic response (*viz.* mammary adenocarcinoma 13672- NF and PAM-CTX sublines and human breast tumors with differing metastatic potential, p53 and Her 2-neu expression),

Specific hypotheses to be tested include: a) that breast tumor pH may be measured accurately, and reproducibly, with useful temporal resolution using ^{19}F MR; b) that CF_3-pyridoxol will provide enhanced pH measurement in breast tumors, and c) that tumors exhibiting different therapeutic response exhibit significant differences in pH regulation. We believe this research will enhance the understanding of tumor pH dynamics, and promises a new prognostic tool to optimize therapy for individual breast tumors.

Body

While we continue to make progress towards the goals of this program, we have experienced some delays. In February this year, Dr. Pieter Otten, who had been recruited in Year 1 as the synthetic chemist moved to an exciting new position with Chembridge Pharmaceuticals in San Diego, CA. The training and research opportunities gained through his participation in this project were of considerable value to his securing the position, but it has caused a delay in our synthetic efforts. We have now recruited an excellent replacement synthetic chemist, Dr. Jianxin Yu (CV appended- item 1), who started in August and is making progress towards the synthetic targets. I have also recruited a second chemist, who will join the laboratory at the start of November. Dr. Wei-na Cui's (CV appended- item 2) primary tasks will be the NMR studies of tumor transmembrane pH and biodistribution and pharmacokinetics of chemotherapeutic drugs. The presence of two chemists in Year 3 will allow us to accelerate studies to compensate for delays. A further delay was caused by failure of both the 4.7 T and 400 MHz NMR systems at the Rogers NMR Center in May this year. This precluded any *in vivo* NMR investigations. Both systems are scheduled to be upgraded and functional again by January 2002, facilitating the originally planned investigations. In addition, we are able to use a 600 MHz instrument for interim tests.

Statement of Work

Phase 1 Design, synthesis and evaluation of enhanced ^{19}F NMR pH indicators:

Task 1 Months 1-6: Recruit and train post-doctoral fellow in synthesis of fluorinated vitamin B6 analogs

While we had recruited a chemist in year 1, he moved on to industry. We have now recruited an outstanding replacement organic synthetic chemist, Dr. Jianxin Yu (curriculum vitae appended; item 1). Dr. Yu has initiated syntheses and designed new synthetic strategies. He has refined synthetic approaches to improve yields and reaction efficiency and is becoming proficient in high field NMR to characterize the new molecules.

Task 2 Months 3-9 Synthesize CF_3-pyridoxol

Our first CF_3-pyridoxol ^{19}F NMR pH indicator was synthesized in year 1, as proposed in the grant application. The final synthetic route was slightly modified. A draft manuscript describing the synthetic approach is appended (Item 3) and will be submitted to BioOrganic Medicinal Chemistry letters in the near future. Dr. Yu has proposed a new synthetic strategy to create di-CF3 pyridoxols (Appendix item 4).

Task 3 Months 6-12 Synthesize CF_3-pyridoxol derivatives modified at 4, 5 and 2 position

Incorporation of an internal chemical shift reference standard can enhance utility of pH reporter molecules. In year 1 we synthesized a derivative of 6-fluoropyridoxol incorporating a 5-SCF_3 group. However, this indicator tended to precipitate at neutral pH and exhibited very broad lines, which were of little use for NMR studies of pH. We proceeded to synthesize a second molecule incorporating a trifluoroacetamido group ($NCOCF_3$) (Appendix 5). This produced narrow lines, but the spectrum is complicated by additional NMR resonances, which we attribute to a second molecular conformation (cis vs. trans.). While this indicator may not be ideal, it could be useful and we are testing it further Appendix item 6 shows titrations).

Task 4 Months 3-12 Characterize new pH indicators, *e.g.*, ^{19}F NMR titrations, high resolution mass spectrometry, ^{1}H, ^{19}F and ^{13}C NMR structural analysis

We have now performed additional titration curves, *e.g.*, on the trifluoroacetamido derivative (Appendix 5), which showed good pH sensitivity and useful pKa, but complexity due to isomers. Other materials are undergoing spectral analysis in preparation for publication. We have also identified an additional class of pH reporter molecule (fluoro nitro phenols), which show strong 19F NMR response to pH and may be useful in vivo (Appendix item 6).

Task 5 Months 6-12: Evaluate molecules in plasma and whole blood

Initial molecules were tested in year 1 and further material will be evaluated as they are synthesized.

Task 6 Months 9-15: Scale up synthesis of most promising molecules

CF_3-pyridoxol was produced in 300 mg quantity. Other materials will be scaled up as needed.

Task 7 Months 9-18: Evaluate most promising molecules in perfused heart model

To be undertaken, by Dr. Cui upon her arrival this year.

Task 8 Month 12: Prepare reports and manuscript.

Report provided. Draft manuscripts appended.

Phase 2 <u>Evaluation of optimal pH indicator *in vivo*:</u>

Task 9 Month 9-18: Evaluate best molecule in breast tumor subline 13672NF (6 tumors)

i: Surgically create pedicles for tumor implantation
ii: Implant tumors and allow to grow to 1 cm diameter
iii: Examine pharmacodynamics of pH indicators *in vivo*; measure baseline pH; verify validity of measurements using ^{31}P NMR and electrodes.

Tumors have been prepared. In vivo assessments will be undertaken by Dr. Cui, as soon as the new MRI systems are functional.

Tasks 10 –15

Will be undertaken on an accelerated basis during coming year.

Key Research accomplishments

- Recruited and trained replacement synthetic chemist
- Successful synthesis of first CF_3-pyridoxol pH indicator.
- Successful synthesis ^{19}F NMR gene reporter molecule.

Reportable outcomes

- Two draft manuscripts (Appendix items 3 and 5)
- Chemist trained for industry
- We successfully competed for an NCI P20 In vivo Cancer Cellular and Molecular Imaging Center Planning grant. The concepts established under the auspices of this DOD BrCa IDEA award were a significant factor is making a successful application. In particular, the spin off technology, identifying gene reporter molecules could have far reaching influence on gene therapy. Further details of this grant and research program may be found on our new web site (http://cip.swmed.edu/ICMIC/).

Conclusions

- CF_3 analogues of F-pyridoxine pH indicators can be synthesized and, as predicted, show a sensitivity ~ 1.5 ppm. ^{19}F resonances are sufficiently narrow to allow useful NMR discrimination of pH. Signal to noise is dramatically improved by the presence of three identical "F" atoms as opposed to a single atom.
- Further experiments are required to modify cellular conditions is order to stimulate uptake of the new class of molecule into cells. This may involve synthesis of further derivatives, which are readily taken up, or manipulation of cellular environment.
- By analogy with the pH reporter molecules originally proposed in this work, we are able to assay gene expression using appropriate ^{19}F NMR reporter molecules.

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Appendix item 1

RESUME

I. GENERAL INFORMATION

Name in Full : Jian-Xin Yu
Sex : Male

PII Redacted

[redacted] : [redacted]

[redacted] : [redacted]

[redacted] : [redacted]

II. EDUCATION BACKGROUND

09/1992~07/1995 : Doctoral student in Department of Organic Chemistry, School of Pharmaceutical Sciences, Beijing Medical University under the direction of Prof. Meng-Shen Cai, Major in Organic Chemistry and received Ph.D. degree in 1995. The Doctorate Thesis: "Synthesis of Some Fragments of Antigenic Polysaccharides".

09/1985~07/1988 : Graduated student in Department of Chemistry, Xinjiang University under the direction of Prof. Yu-Ting Liu, Major in Organic Chemistry and received M.S. degree in 1988. The Master Thesis: "The Oxidation of Secondary Hydroxyl Group in Monosaccharides and Synthesis of Some Branched Monosaccharides".

09/1981~07/1985 : Undergraduate student in Department of Chemistry, Xinjiang University, Major in Chemistry and received B.S. degree in 1985.

III. WORKING EXPERIENCE

06/2000~Present : Faculte de Pharmacie, Department de Pharmacochimie Moléculaire/Glucides, Université Joseph Fourier-Grenoble 1, Research Associate.

02/2000~05/2000 : National Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Beijing Medical University, Visiting Professor, Researching on the Synthesis and Characteristics of Heterocyclic Compound Linked to Carbohydrates.

07/1995~01/2000 : Department of Chemistry, Xinjiang University, Associate Professor, Teaching Courses (Lecturing & Laboratory): Organic Synthesis, Advanced

Organic Chemistry, Organic Analytical Chemistry, Modern Carbohydrate Chemistry.

07/1988~09/1992 : Department of Chemistry, Xinjiang University, Lecturer, Teaching Courses (Lecturing & Laboratory): Organic Chemistry, Organic Analytic Chemistry, Natural Compounds Chemistry.

IV. RESEARCH AREAS & CURRENT PROJECTS

1. Synthesis of Multivalent Saccharide-branched β-Cyclodextrins.
2. Synthesis of Heterocyclic Compound Linked to Carbohydrate & Studies on the Action of Carbohydrate on the Selectivity of Chiral Heterocyclic Compound Formation.
3. Synthesis of Some Fragments of Antigenic Oligosaccharide.
4. Studies on Some New Organic Reactions & the Application of the Known Organic Reactions in the Carbohydrate Synthesis.
5. Synthesis of Bioactive Heterocyclic Compounds.
6. Separation & Structure Analysis of the Bioactive Chemical Ingredients from Xinjiang Natural Plants.

V. HONORS

1. The Outstanding Youth Chemical Prize from Chinese Chemical Society in 1992.
2. The Research Project Achievement Award from Xinjiang University in 1997.
3. The Outstanding Academic Thesis Prizes from the Government of Xinjiang Uygur Autonomous Region in 1996 & in 1998.
4. The Research Project Achievement Award from the Government of Xinjiang Uygur Autonomous Region in 1999

VI. LANGUAGE ABILITY

Chinese, English (Listening, Speaking, Reading & Writing).

VII. COMPUTER APPLICATION

Skillful in Windows, Word, Chemwin & Some Other Softwares.

VIII. AFFILIATION

Chinese Chemical Society.

VIIII. SELECTED PUBLICATIONS

35. Jian-xin Yu, Su-na Zhang, Zhong-jun Li, Wen-jie Lu, Yu-ting Liu, Meng-shen Cai, Stereoselective Synthesis of 2-C-(1,2:4,5-Di-O-Isopropylidene--D - Ribohex-2-ulo Pyranose)-5-Substituted-1,3,4-Oxadiazoles, *Carbohydr. Lett.*, 2000 (Submitted).
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28. Jian-xin Yu, Fang-ming Liu, Yan-ping Li, Liang Chen, Yu-ting Liu, Cong Liu, Ming Li, Meng-shen Cai, Synthesis of 1-Aroyl-4-[1-N-2,3,4,6-Tetra-O-Acetyl-*β*-D-Gluco- pyranosyl] Thiosemicarbazides and Studies on Their Cyclization, *Chem. J. Chin. Univ.*, 2001(in press).

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3. Jian-xin Yu, Yu-ting Liu, The Oxidation of the Secondary Hydroxyl Group in Monosaccharides, *Chemistry*, 1992, (5), 1~5.
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1. Rui Wang, Yao-zu Chen, Jian-xin Yu, Studies on Chemical Ingredients of *Aconitum Karakolicum var. Patentipilum*, *Chem. J. Chin. Univ.*, 1988, **9**(7), 740~ 742.

X. REFERENCES

1. Prof. Yu-ting Liu, Department of Chemistry, Xinjiang University, Wulumqi 830046, China, Tel: 0086-991-2862753 ext. 2677, Fax: 0086-991-2862006, E-mail: Liuzhu0370@sina.com.
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3. Prof. Ji-De Wang, Department of Chemistry, Xinjiang University, Wulumqi 830046, China, Tel: 0086-991-2862753 ext. 3141, Fax: 0086-991-2862006.

Appendix Item 2

WeiNa Cui

Curriculum vitae

Department of Medicinal Chemistry
School of Pharmaceutical Sciences,
Peking University Health Science Center
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OBJECTIVE

I am writing this letter applying for the post-doctoral position in Medicinal Chemistry.

5 EDUCATION

September, 1997-June, 2000 Peking University, Beijing, P. R. China
Ph.D. of science majoring in Medicinal Chemistry
1995- 1997 Peking University, Beijing, P.R China
Master of Science majoring in Medicinal Chemistry
1991-1995 Peking University, Beijing, P.R China
Bachelor of science majoring in Medicinal Chemistry

6 RESEARCH EXPERIMENCES

1.Design and Synthesis of Pentapeptides
GH can act on skeleton directly or indirectly. Growth hormone releasing peptides (GHRPS) are small synthetic peptides characterized by a high potency in stimulating GH releasing in vivo and vitro. So we designed and synthesized a series of pentapeptides to prevent and treat osteoporosis.
2.Design and Synthesis of Linkers constituted with Pentapeptides and Estrogen
Estrogen can also promote bone formation. There is "promissive effect" between peptides and steroids in linkers constituted with peptides and steroids. Therefore we designed and prepared a series of compounds, which are linkers of pentapeptides and estone or estradiol.
3. Effect of Pentapeptides and Linkers on Osteoporosis and the studies of their SAR
In order to evaluate the effect of above-mentioned compounds on osteoporosis, predinisone was used to induce osteoporosis, at the same time estone and estradiol were regarded as positive controls. Seven indexes were measured, including weight of ratsd r y weight of femursas h weight of femursca l cium content of femurs phosphorous content of femurss e rum concentration of calciumse r um concentration of alkaline phosphatase. The results indicated that some of the compounds could prevent bone loss, showing potential activity. The studies of SAR showed that linkers revealed predominant pharmacological action than peptides and estrogen.

7 WORKING EXPERIENCES

Oct.2000- present Venture Pharmacy
As a project manager, I am engaged in new drug R&D, including heterocyclic compounds st e roids and peptides etc.
Sept.1995-July. 1997 Department of Medicinal Chemistry, Beijing Medical University
Synthesis on Melatonin
The object of this job is to furnish a piece of rather perfect synthetic route for industrialization of Melatonin. After fulfilling it, I completely and systematically knew about how to exploit drugs and apply them to industrial production.
Advisor: professor Li An-liang
March1995-June 1995 Department of Medicinal Chemistry, Beijing Medical University
Measuring Hydrophobic Parameter of Compounds by HPLC
Advisor: professor Zou An-qing

COMPETENCE and LAB SKILLS

Solid-phase peptide synthesis and liquid-phase peptide synthesis
The synthesis of steroids and heterocyclic compound

Computer Aided Drug Design

NMR, mass spectrometry, ESR, HPLC and related analytical methods

8 ENGLISH LANGUAGE ABILITY

Powerful capability of speaking, reading, writing and listening English, Be skillful in reading special literature and intercoursing fluently with English.

9 PUBLICATION

1.Studies on the Linkers constituted with Peptides and Steroids , 2000 in press

2.Studies of the GHRPS on Osteoporosis, 2000 in press

3.Comparing Teaching Material of Steroids Medicinal Chemistry 1996

4.Mensurating o/w Distributing coefficient of 8-Chloro-Adenosine and its correlative compounds 1995

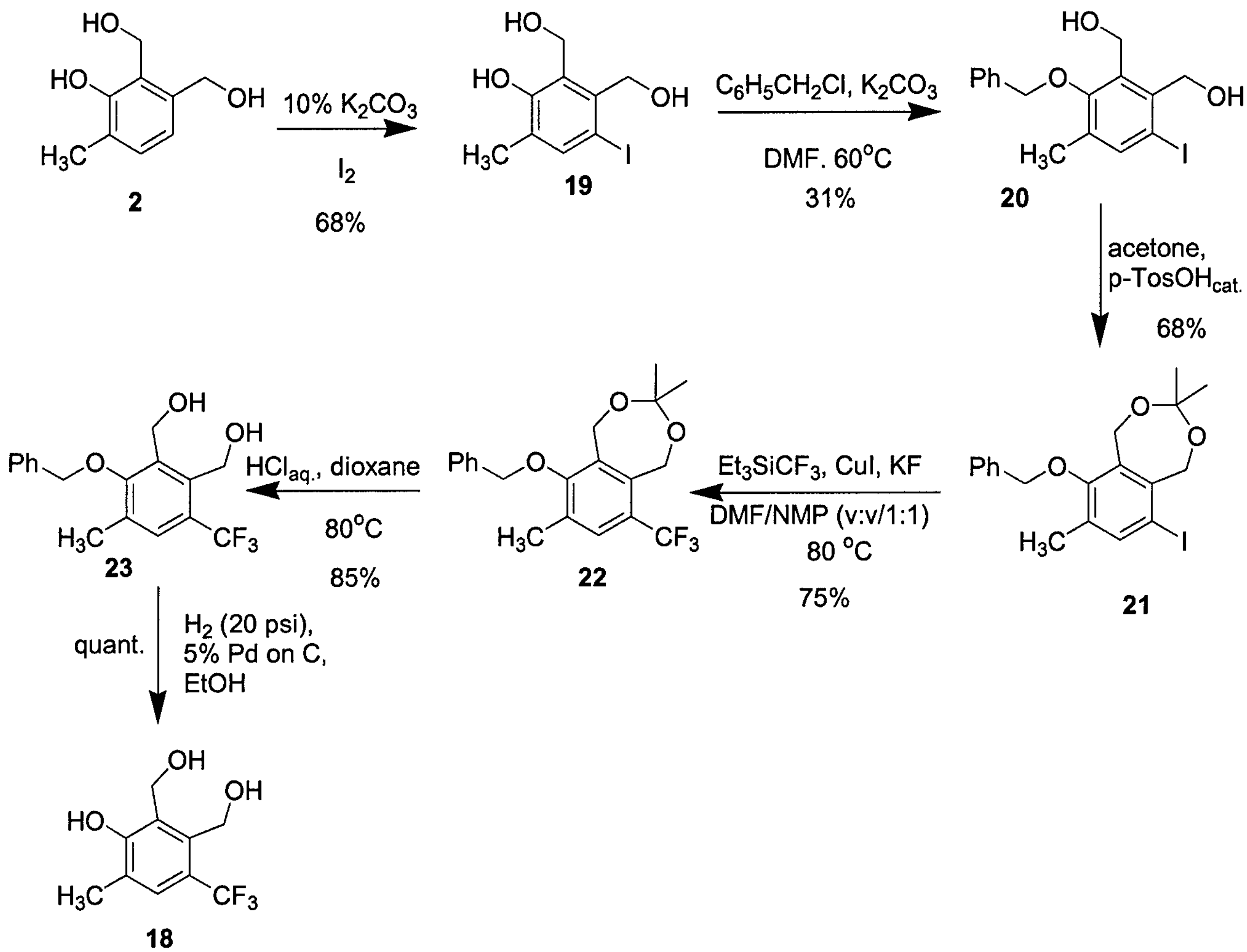
REFERENCER

1. Peng Shi-qi, Professor of Medicinal Chemistry, Dean of School of Pharmaceutical sciences, Peking University Health science Center, Beijing PR China
2. Li An-liang, Professor of Medicinal Chemistry, Peking University Health science Center, Beijing PR China
3. Zou An-qing, Professor of Medicinal Chemistry, Peking University Health science Center, Beijing PR China

Appendix 3
Development of enhanced pH reporter molecule: trifluoromethyl pyridoxol
Draft manuscript for submssion to Bioorganic Medicinal Chemistry letters
6-CF_3POL

The introduction of a trifluoromethyl group at an aromatic ring can be achieved by a substitution reaction on an aryl iodide with trifluoromethyl copper ("$CuCF_3$") [7]. Urata [8] described a convenient method for the *in situ* generation of $CuCF_3$ by a metathesis reaction of commercially available Et_3SiCF_3 with CuI in the presence of an aryl iodide which serves as the substrate in the trifluoromethylation reaction. For the synthesis of 6-(trifluoromethyl)pyridoxine (**18**), we required access to 6-iodopyridoxine (**19**) and protection of its alcohol groups. The three-step halogenation of pyridoxine (**2**)- via 6-aminopyridoxine (**4**), followed by a Sandmeyer reaction - has been very successful for the synthesis of our ^{19}F NMR pH indicator 6-fluoropyridoxine (**1**) [1, 2, 9, 10]. We decided, however, to attempt the direct iodination of pyridoxine (**2**) using a procedure previously described for the iodination of 3-hydroxypyridine in aqueous base [11]. The reaction of pyridoxine (**2**) with iodine was carried out in 10% K_2CO_3 generating the pyridoxine anion, which is the actual reactive species. Acidification of the reaction mixture resulted in the precipitation of 6-iodopyridoxine. **19** could be obtained pure in a yield of 68% by filtration. Attempts to protect all three hydroxy groups of **19** at once as their respective benzyl ethers (NaH, benzyl chloride, DMF) failed and led to unidentifiable product mixtures. Instead, a two step approach was used. Firstly, the more acidic phenolic hydroxy group was protected as its benzyl ether by using milder basic reaction conditions: K_2CO_3 in warm DMF in the presence of benzyl chloride, which afforded benzyl ether **20** in a modest 31% yield. Next, the two remaining hydroxy groups were protected together in the form of acetone acetal **21**. Acetal **21** was obtained in 68% yield by the reaction of **20** in acetone in the presence of a catalytic amount of *p*-toluenesulfonic acid followed by chromatographic work-up.

We were gratified to see that Urata's trifluoromethylation methodology for phenyl iodides could be extended to a more sterically hindered iodopyridine ring such as our protected iodopyridoxine **21**. Thus, reaction of **21** in a mixture of DMF and NMP (v/v:1/1) in the presence of CuI, Et_3SiCF_3 (b.p. 41 °C), and KF in a sealed tube at 80 °C led to complete conversion of **21** and gave protected 6-(trifluoromethyl)pyridoxine **22** in a yield of 75% after purification by flash chromatography. Subsequent removal of the acetal protection of the aliphatic hydroxy groups was carried out in a mixture of dioxane and 1M HCl at 80 °C. Benzylated (trifluoromethyl)pyridoxine **23** precipitated upon removal of the dioxane from the reaction mixture and was isolated in a yield of 85 % after filtration. The synthesis of the desired 6-(trifluoromethyl)pyridoxine (**18**) was finalized by deprotection of the phenolic hydroxy group by hydrogenolysis of the benzylether **23,** which proceeded in near quantitative yield.



Titrations

The [19]F NMR signal of 6-CF_3POL in water shows one sharp line. Using CF_3COONa as a reference, the chemical shift moves downfield from 15.16 ppm (at pH = 2.18) to 16.74 ppm (at pH = 11.08) upon increasing pH. Thus, the chemical shift sensitivity of 6-CF_3POL is a factor 6 less than 6-FPOL. This observation can be readily explained by the fact that in 6-CF_3POL the three reporting fluorine atoms are not in direct communication through resonance with the sensor: the phenolic OH group. The pK_a of 6-CF_3POL was determined at 6.7 compared to 8.2 for 6-FPOL. Apparently, the stronger electron-withdrawing nature of the CF_3 group causes this marked increase in acidity, which makes 6-CF_3POL a better probe for physiological measurements in more acidic tumor tissue. No significant line broadening of the ^{19}F resonance was observed at or around the pK_a value of 6-CF_3POL. In our opinion, this means that despite the reduced chemical shift range, 6-CF_3POL will be useful as a sensitive NMR pH indicator.

In fresh rabbit blood, at 22 °C, 6-CF_3POL shows only one resonance after incubation at 37 °C for 4 hours (2 mg of CF_3POL in 0.5 ml of blood). This observation clearly indicated that no significant amounts, if any at all, of 6-CF_3POL accumulated in the available red blood cells to allow the detection of an intracellular signal. Raising the NMR probe temperature to 37 °C did not result in the appearance of an additional, intracellular, signal. This is in sharp contrast to our observations for 6-FPOL and its amino derivative 6-FPAM ($pK_a = 7.05$) which both report an intracellular ^{19}F NMR signal within minutes after mixing with fresh blood. In this particular blood sample, CF_3POL displayed a resonance at 16.49 ppm, which corresponds with an extracellular pH of 7.47 after applying the Henderson-Hasselbach equation. The pH of the sample was determined at 7.39 by a pH electrode. At this point it is not clear whether 6-CF_3POL does not penetrate the cell membrane at all, or whether it is rapidly expelled again from the cell by a transporter after entering the cell by either diffusion and/or active transport.

Methods

6-Iodopyridoxine ( ). To a suspension of 4.25 g (25 mmol) pyridoxine in 75 ml water was added 6.9 g (47 mmol) K_2CO_3 resulting in complete dissolution of all solid material. Subsequently, 6.3 g (25 mmol) iodine was added in one portion followed by stirring for 1 h at room temperature. To the dark brown reaction mixture, 400 mg Na_2SO_3 was added resulting in a yellow clear solution. The reaction mixture was subsequently quenched with concentrated HCl to a pH of 3 and the precipitate was isolated by filtration over a Büchner filter and dried *in vacuo* over sodium hydroxide to give 4.98 g (68 %) of a yellow powder. ^1H NMR (dmso-d_6) δ 2.30 (s, 3H), 4.55 (d, J = 6 Hz, 2H), 4.79 (s, 2H), 5.07 (t, J = 6 Hz, 1H), 5.8 (br, 1H), 9.5 (br, 1H).

6-Iodo-3-*O*-benzylpyridoxine ( ). To a solution of 9 g 6-iodopyridoxine (31 mmol) in 30 ml dry DMF under argon were added 18 g K_2CO_3 (123 mmol) and 12 ml benzylchloride (104 mmol). The reaction mixture was stirred at 60 °C overnight, allowed to cool to room temperature, and filtered over a Büchner filter. The filtrate was poured into 200 ml water and extracted three times with 200 ml EtOAc. The combined organic layers were dried on $MgSO_4$, filtered, and evaporation of the solvent *in vacuo* gave a dark solid, which was triturated with 50 ml diethyl ether. Filtration over a glass filter and drying *in vacuo* over sodium hydroxide gave 4.2 g (35 %) of a pink solid. ^1H NMR (dmso-d_6) δ 2.42 (s, 3H), 4.68 (s, 2H), 4.70 (s, 2H), 4.94 (s, 2H), 7.40-7.54 (m, 5H).

6-Iodo-3-*O*-benzyl-α^4,α^5-*O*-isopropylidenepyridoxine ( ). To a solution of 4.2 g (11 mmol) 6-iodo-3-*O*-benzylpyridoxine ( ) in 60 ml acetone was added 1.2 g *p*-toluenesulfonic acid. The reaction mixture was stirred at room temperature for two days after which another 50 ml of acetone and 1 g of *p*-toluenesulfonic acid were added, followed by an additional stirring for 16h. The reaction mixture was neutralized by the addition of 50 ml water and 3 g K_2CO_3. The acetone was removed *in vacuo* and the remaining aqueous layer was extracted three times with 150 ml EtOAc. The combined organic layers were dried on $MgSO_4$, filtered, and evaporation of the solvent *in vacuo* gave a dark oil which was further purified by flash chromatography (eluent: 10% EtOAc in hexane) to give 3.2 g (68 %) of a white solid. ^1H NMR ($CDCl_3$) δ 1.45 (s, 6H), 2.46 (s, 3H), 4.77 (s, 2H), 4.78 (s, 2H), 4.79 (s, 2H), 7.36-7.42 (m, 5H).

3-*O*-benzyl-α^4,α^5-*O*-isopropylidene-6-(trifluoromethyl)pyridoxine ( ). To a solution of 760 mg (1.8 mmol) 6-Iodo-3-*O*-benzyl-α^4,α^5-*O*-isopropylidenepyridoxine ( ) in a mixture of 2 ml DMF and 2 ml NMP in a 15 ml glass pressure tube were added 510 mg (2.7 mmol) CuI and 125 mg (2 mmol) KF. The well-stirred solution was kept under an argon flow for 30 min followed by the addition of 500 μl Et_3SiCF_3 (2.7 mmol) and the tube was sealed. The mixture was stirred at 80 °C for 16 h and allowed to cool to room temperature. The clear, dark brown reaction mixture was poured into 100 ml water and the resulting white suspension was extracted three times with 200 ml diethyl ether. The combined organic layers were washed three times with 100 ml water, dried on $MgSO_4$, filtered and evaporation of the solvent *in vacuo* gave a dark oil which was purified by flash chromatography (eluent: 10% EtOAc in hexane) to give 496 mg (75 %) of a colorless viscous oil. ^{1}H NMR ($CDCl_3$) δ 1.48 (s, 6H), 2.48 (s, 3H), 4.78 (s, 2H), 4.79 (s, 2H), 4.80 (s, 2H), 7.40-7.50 (m, 5H).

3-*O*-benzyl-6-(trifluoromethyl)pyridoxine ( ). To a solution of 1.5 g (4.0 mmol) 3-*O*-benzyl-α^4,α^5-*O*-isopropylidene-6-(trifluoromethyl)pyridoxine ( ) in 10 ml 1,4-dioxane was added 5 ml 1 M HCl. The turbid mixture was heated to 80°C, eventually became clear, and stirring was continued for 4 h at this temperature. TLC (eluent: 25% EtOAc in hexane) indicated that all starting material had been consumed. After the reaction mixture was allowed to cool to room temperature, all volatile materials were removed *in vacuo* which resulted in the formation of a white precipitate. The crude product was evaporated to dryness, suspended in 10 ml water and filtered. The residue was dried *in vacuo* on NaOH to give 1.1 g (85 %) of a white powder. ^{1}H NMR (dmso-d_6) δ 2.49 (s, 3H), 4.72 (s, 4H), 5.00 (s, 2H), 7.40-7.45 (m, 3H), 7.52-7.54 (m, 2H).

6-(trifluoromethyl)pyridoxine (6-CF3POL,). To a suspension of 1.0 g (3.1 mmol) of 3-*O*-benzyl-6-(trifluoromethyl)pyridoxine ( ) in 100 ml ethanol was added 500 mg of 5% Pd on C. The mixture was hydrogenolyzed for 16h under 25 psi after which TLC (eluent: 50% EtOAc in hexane) indicated complete consumption of the starting material and the formation of one single product. The reaction mixture was filtered over Celite and the solvent was evaporated *in vacuo* to give 731 mg (99 %) of an off-white solid. ^{1}H NMR (dmso-d_6) δ 2.37 (s, 3H), 4.55 (d, J = 0.9 Hz, 2H), 4.84 (s, 2H).

Acknowledgments

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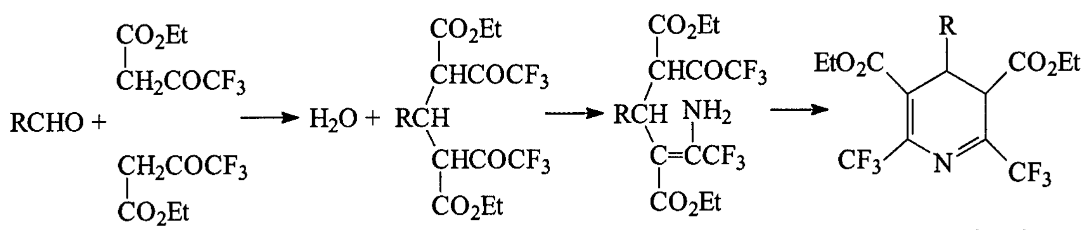
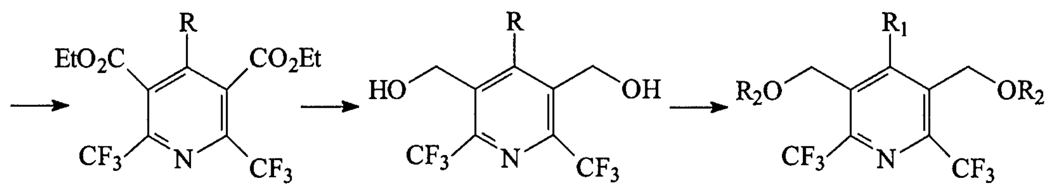
Appendix Item 4

Syntheses of Di-trifluoromethyl containing pyridoxol Analogues as Potential ^{19}F MRI and NMR Agents for Breast Tumor pH Indicator Studies

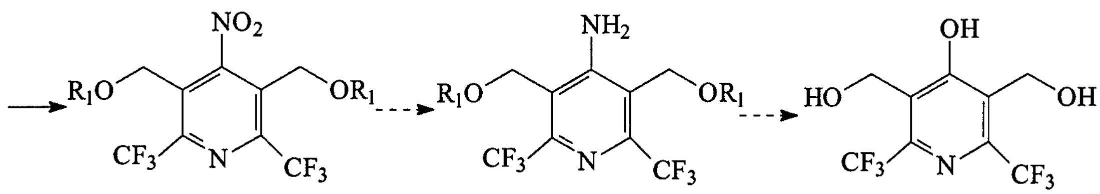
A. Synthesis of Di-trifluoromethyl containing pyridoxol Analogues Through Claisen Condensation Reactions



R = H, CH_3, CH_2CH_3 etc

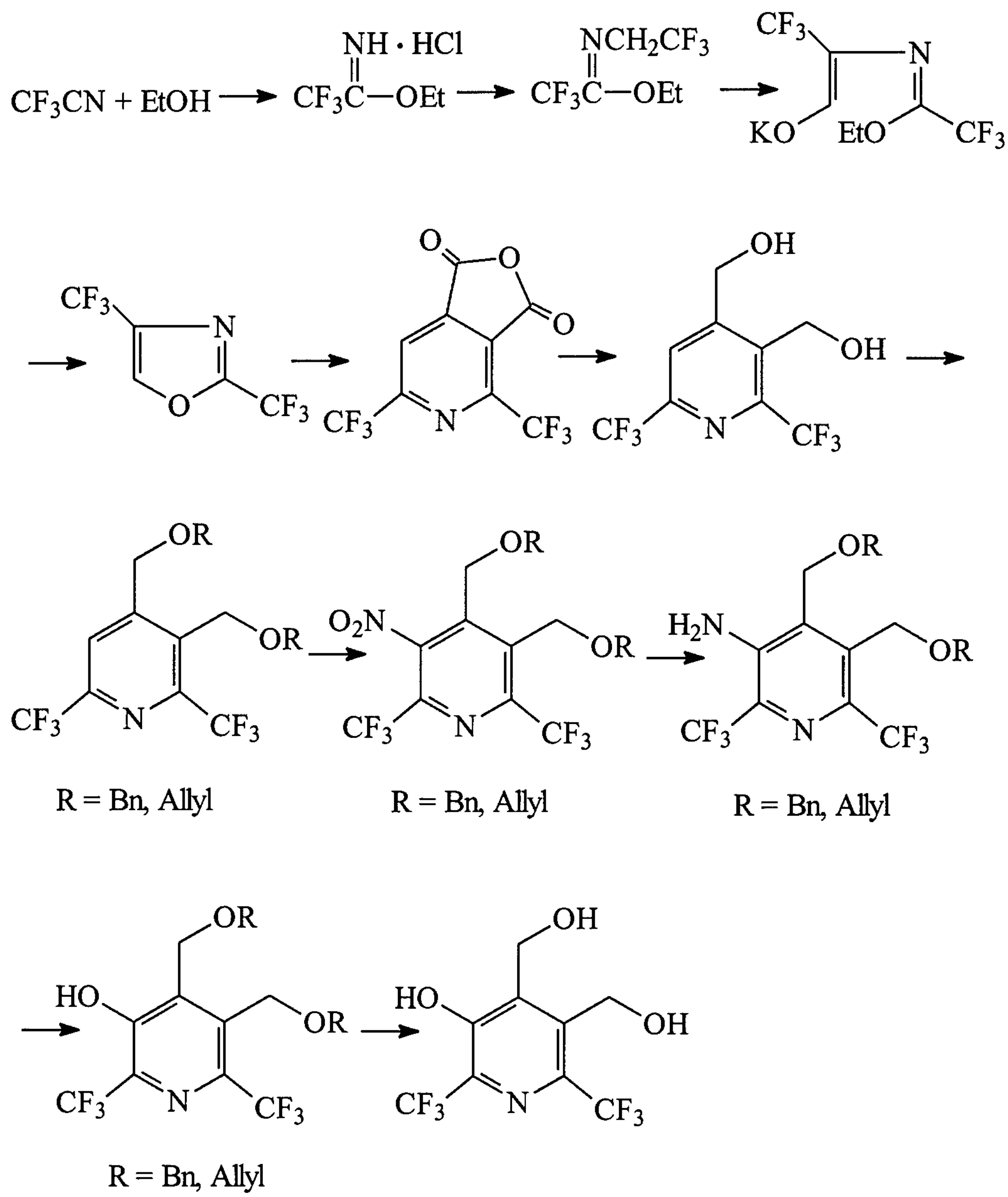


R = H, CH_3, CH_2CH_3 etc   R = H, CH_3, CH_2CH_3 etc R_1 = H, R_2 = Ac, Bn, Allyl



R_1 = Ac, Bn, Allyl R_1 =H, Ac, Bn, Allyl

B. Synthesis of Di-trifluoromethyl containing pyridoxol Analogues Through Oxaole Methods



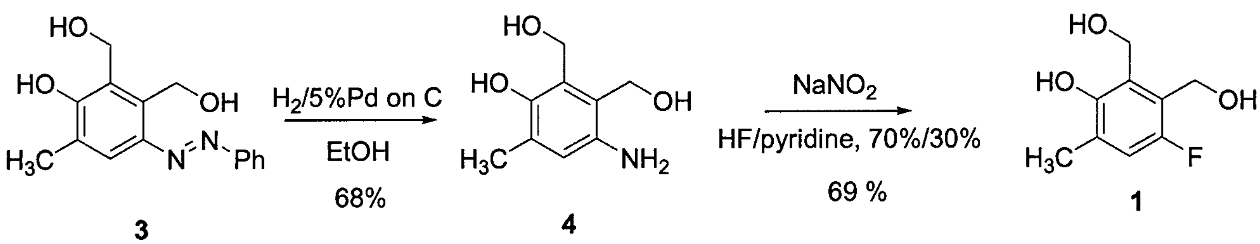
Appendix 5

Draft manuscript for submission to Bioorganic Medicinal Chemistry Letters

Development of novel pH indicator incorporating internal chemical shift reference.

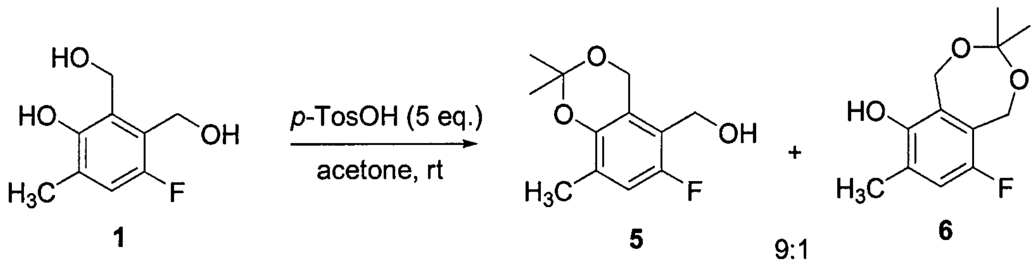
Synthesis

Our first task at hand was to improve the earlier reported synthesis of 6-fluoropyridoxine (6-FPOL, **1**). 6-FPOL can be synthesized in three steps starting from the commercially available hydrochloric salt of pyridoxine (vitamin B_6, **2**). First, using the procedure of Katritzky *et al.*[3], **2** was converted to 6-phenyldiazopyridoxine (**3**). Subsequent hydrogenation of the phenyldiazo moiety over palladium on carbon [4] gave the corresponding 6-aminopyridoxine (**4**) in yields up to 68%. A high degree of purity of the intermediate 6-aminopyridoxine proved to be essential for a successful fluorination of the pyridine ring to form 6-FPOL. We therefore devised a simpler, yet efficient purification method for **4** by triturating the crude 6-aminopyridine with diethyl ether (see: Experimental). The synthesis of 6-fluoropyridoxine (**1**) was finalized by a Schiemann reaction. We found that 40% HBF_4, originally described[2] as the fluoride source for this reaction, gave no isolable product. The use of 70% HF in pyridine as both reaction solvent and fluoride source gave, after neutralization of the reaction mixture and extractive work-up with diethyl ether, the desired 6-FPOL (**1**) in a pure state in yields up to 69 %.

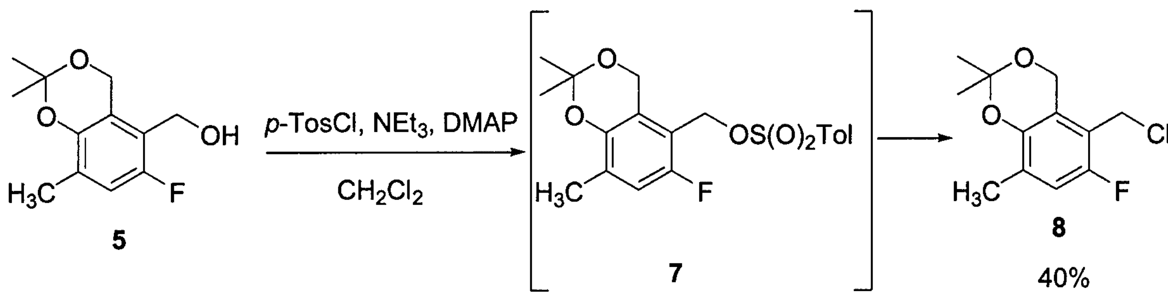


The pyridoxine skeleton offers several reactive sites for the introduction of other functional groups. We chose to use the 5-hydroxymethylene group for the introduction of an internal ^{19}F NMR reference. The synthetic potential of this site has recently been described by us[3] and others[2] taking advantage of the fact that [1] this hydroxyl group can be readily differentiated from the other hydroxyl groups in **1**. Its synthetic isolation can be achieved [4] by selectively protecting the phenolic OH and its neighboring 4-hydroxymethylene group as an acetone acetal. In addition, the position *meta* with respect to the reporter group (3-hydroxy group) and the one-carbon tether is expected to desensitize the chemical shift of the internal reference to pH changes. We decided to introduce at this position the more lipophilic (trifluoromethyl) thioether group and a 2,2,2-trifluoroacetamido moiety, respectively because of their ready synthetic accessibility.

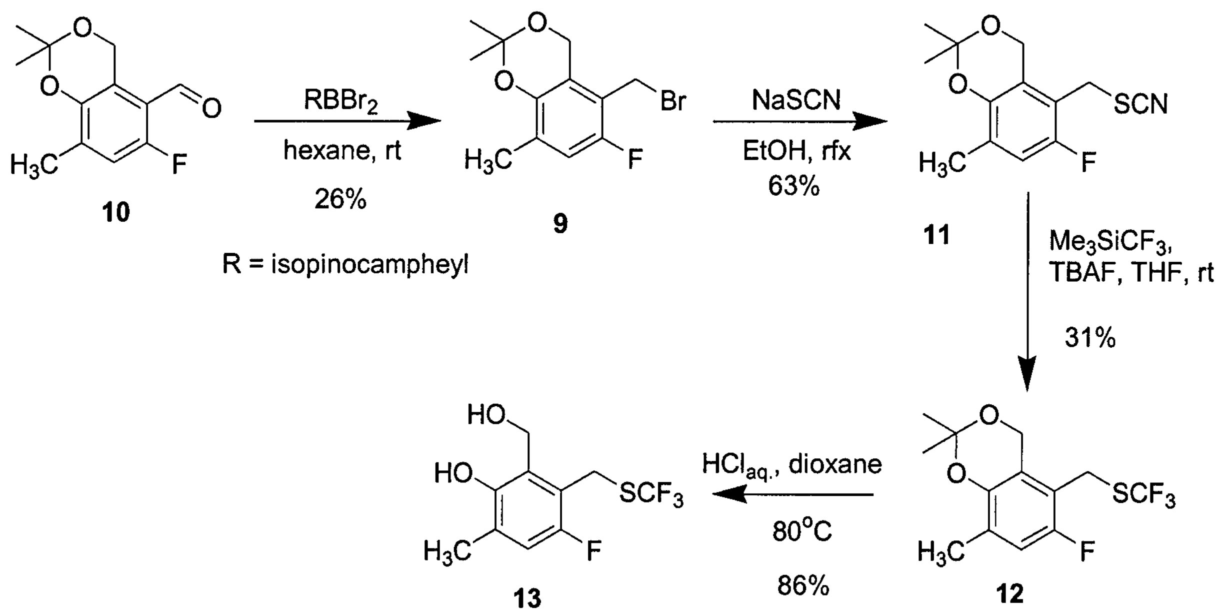
The acetonation of **1** was carried out in acetone in the presence of a five-fold excess of *p*-toluenesulfonic acid. In our hands, exclusive formation of the desired α^4,3-*O*-isopropylidene isomer **5** did not occur though as under these reaction conditions, up to 10% of the regioisomeric acetal **6** could be observed in the ^1H NMR spectrum of the crude product. The presence of regioisomer **6**, however, did not interfere with the purification of subsequent products and the crude **5** was used as such.



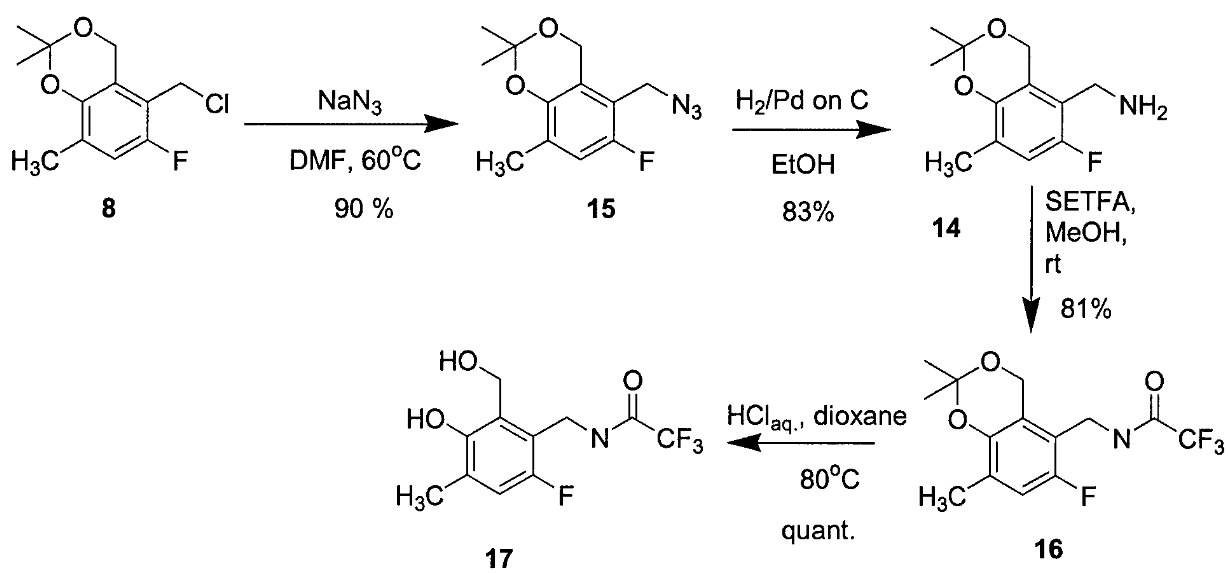
Tosylation of alcohol **5** would allow the introduction of new functionality through nucleophilic substitution reactions. Standard tosylation procedures, however, did not provide the desired sulfonic ester **7**. Instead, the use of *p*-toluenesulfonyl chloride and triethylamine as the proton acceptor in CH_2Cl_2, in the presence of a catalytic amount of DMAP, led to the isolation of pyridoxyl chloride **8** in a yield of 40%. It is believed that the highly reactive tosylate **7** serves as an intermediate that is rapidly converted to chloride **8** by HCl (or its triethylamine salt).



Substitution on chloride **8** with sodium thiocyanate in refluxing ethanol did not proceed cleanly and the resulting extensive chromatographic purification led to significant loss of product. It was decided therefor to synthesize the more reactive pyridoxyl bromide **9**, but unfortunately attempts to directly convert alcohol **5** to bromide **9** failed. The solution to this problem was found in the reductive bromination of aldehyde **10** according to a recently described procedure by Kabalka *et al.* [5]. The required aldehyde **10** was obtained by oxidation of **5** with MnO_2 according to a literature procedure[2]. Treatment of **10** with isopinocampheyl borondibromide, generated *in situ* from dibromoborane and α-pinene, furnished pyridoxyl bromide **9** in a yield of 26% after chromatography. As was anticipated, pyridoxyl thiocyanate **11** was readily obtained pure in 63% yield by reaction of **9** with NaSCN in refluxing ethanol followed by chromatographic work-up. The desired trifluoromethyl reference group was finally introduced using Ruppert's reagent[6] [6] (Me_3SiCF_3) for the conversion of thiocyanate **11** to (trifluoromethyl)thio ether **12** which was obtained pure, after chromatography in 31% yield. Final deprotection of the acetone acetal in **12** was achieved with aqueous HCl in dioxane to give our first targeted ^{19}F NMR indicator with an internal reference: **13**.



The introduction of a 2,2,2-trifluoroacetamido moiety can be achieved by acylation of pyridoxylamine **14** with (*S*)-ethyl trifluoroacetate (SETFA). Starting from pyridoxyl chloride **8**, the required pyridoxyl amine **14** could be obtained in two steps. A nucleophilic substitution on chloride **8** with NaN_3 in DMF gave pyridoxyl azide **15** in 90% yield after chromatographic purification. Subsequent hydrogenolysis of azide **15** gave **14** in 83% yield and acylation of **14** with SETFA in MeOH led to the isolation of *N*-pyridoxyl trifluoroacetamide **16** in a yield of 81%. Final deprotection of **16** with aqueous acid proceeded quantitatively to furnish **17**: a new 6-FPOL derivative with a built-in internal reference.



Titrations

The more lipophilic nature of (6-fluoropyridox-5-yl) trifluoromethyl sulfide results in poor water solubility. At neutral pH, no ^{19}F NMR signal could be observed and thus no pH/NMR titration was feasible.

The ^{19}F NMR spectrum of *N*-[6-fluoropyridox-5-yl]-2,2,2-trifluoroacetamide ( ) in water at 22 °C shows four well-separated sharp singlets corresponding to two CF_3 groups and two F substituents at the pyridoxyl ring of the two rotational isomers. Heating the sample to 90 °C does not result in a coalescence of the signals. Thus, rotation around the C-N bond of the trifluoroacetamide moiety at ambient temperatures is slow on the NMR time scale, which can be explained by the strongly electron-withdrawing nature of the CF_3 group. Because of overlap of the signal from CF_3COONa, which we routinely use as a reference for ^{19}F NMR, with the CF_3-signals KF was used as the reference. Both signals for the fluorine substituent at the pyridoxyl ring undergo an upfield shift upon an increase of the pH. The minor isomer shows a chemical shift range of 11.1 ppm between acid and base and the major isomer shows a slightly larger chemical shift range of 11.5 ppm. These observed values are an improvement with respect to the chemical shift sensitivity of 9.72 ppm for FPOL. As might be expected, the shapes of both titration curves are nearly identical, however the curves cross each other at a pH of approximately 7. Interestingly, the pK_a's of both rotational isomers are not identical. The pK_a of the minor isomer was determined at 7.7, whereas the major isomer was found to have a pK_a of 8.1. This indicates that, despite the CH_2 tether between the pyridoxyl ring and the trifluoroacetamide group, isomerization of the latter exerts a subtle, but measurable influence of the acidity of the molecule. Both signals show line broadening when the pH is close to their respective pK_a values. The chemical shifts of both CF_3 signals remain constant during the pH/NMR titration at 43.71 and 43.52 ppm for the minor and major isomer, respectively. No attempts were made to assign the ^{19}F signals to the corresponding rotational isomers.

General Remarks.

All chemicals were used as received except acetone, which was dried on K_2CO_3 prior to use. Thin-layer chromatography was carried out on Sigma/Aldrich TLC plates. Chromatographic purifications were done by flash chromatography using Merck Silica Gel, grade 9385, 230-400 mesh, 60 Å. ^1H and ^{19}F NMR were recorded on a . Chemical shifts are given in parts per million (δ), using TMS as an internal standard for ^1H NMR and CF_3COONa or KF for ^{19}F NMR. Signals are expressed as an s (singlet), d (doublet), t (triplet), m (multiplet), or br (broad).

6-Aminopyridoxine (4). To a suspension of 10.5 g (38 mmol) of phenyldiazoniumpyridoxol **(3)** in 200 ml ethanol was added 4 g of 5% Pd on carbon. The suspension was hydrogenolyed for 16 h at room temperatures at an initial hydrogen pressure of 30 psi. The black suspension was filtered over Celite and the solvent was evaporated *in vacuo* to give an orange solid. The solid material was stirred overnight with 25 ml diethyl ether, filtered over a glass filter and dried *in vacuo* over sodium hydroxide to yield 4.8 g (68 %) of an off white solid.

6-Fluoropyridoxine (6-FPOL, 1). To 50 g HF/pyridine (70% wt HF) cooled with an ice bath, was added 15 g (81 mmol) aminopyridine **(4)** over a 30 min period. Subsequently, 7 g (101 mmol) of $NaNO_2$ was added in small portions over a 1h period. After about half of the $NaNO_2$ was added, gas evolution became less vigorous. The dark brown reaction mixture was stirred for an additional 30 min, followed by 1.5 hours at 65°C. Initially, gas evolution resumed, subsided again after about 15 min after which all solid material had dissolved. The reaction mixture was cooled with a ice bath, diluted with 100 ml water, and carefully neutralized to a pH of 5 with sodium hydroxide pellets (**caution:** adding the hydroxide pellets too quickly and in larger amounts results in an exothermic reaction and a boiling HF-containing mixture. Alternatively, adding a concentrated sodium hydroxide solution allows a more controlled neutralization). The neutralized reaction mixture was filtered over a Büchner filter. The brown filtrate was extracted five times with 200 ml diethyl ether (with which, before each extraction, the filtration residue was thoroughly washed). The combined organic layers were dried on $MgSO_4$, filtered, and the solvent was evaporated *in vacuo* to yield 9.2 g (69 %) of a pink solid which, according to ^1H and ^{19}F NMR reported earlier, was identified as pure 6-FPOL (**1**).

6-Fluoro-α^4,3-*O*-isopropylidenepyridox-5-yl chloride ( ). To a solution of 3.74 g (16.4 mmol) 6-fluoro-α^4,3-*O*-isopropylidenepyridoxol in 12 ml dry CH_2Cl_2 were added 5.7 g (30 mmol) *p*-toluenesulfonyl chloride, 10 ml triethylamine, and 100 mg DMAP. An exothermic reaction ensued and the reaction mixture was cooled for 10 min with an ice bath. A precipitate eventually appeared and the reaction mixture was stirred for 16 h at room temperature. The dark brown reaction mixture, diluted with 75 ml water and an additional 150 ml CH_2Cl_2, was transferred to a separating funnel. The separated organic layer was dried on $MgSO_4$, filtered, and evaporation of the solvent *in vacuo* gave a brown mass which was purified with flash chromatography (eluent: 10% EtOAc in hexane) to yield 1.6 g (40 %) of a white solid. ^1H NMR ($CDCl_3$) δ 1.56 (s, 6H), 2.36 (s, 3H), 4.50 (s, 2H), 4.97 (s, 2H). MS (m/z)

6-Fluoro-α^4,3-*O*-isopropylidenepyridox-5-yl bromide ( ). To a solution of 1.54 g (6.8 mmol) of 6-fluoro-α^4,3-*O*-isopropylidenepyridox-5-al in 25 ml hexane under argon, was added, through a syringe, a freshly prepared solution of isopinocampheyl borondibromide (by refluxing under argon, for 5 h, 7.5 ml of a 1.0 M solution of $HBBr_2{\bullet}Me_2S$ in CH_2Cl_2 with 1.2 ml α-pinene) at room temperature. A white precipitate immediately formed and stirring was continued overnight. The reaction mixture was filtered over a paper filter and the reaction flask was rinsed twice with 75 ml CH_2Cl_2. The combined organic layers were washed once with 100 ml water, dried on $MgSO_4$, filtered, and the solvent was evaporated *in vacuo*. The crude product was further purified with flash chromatography (eluent: 10% EtOAc in hexane) to give 514 mg (26 %) of a white solid. ^1H NMR ($CDCl_3$) δ 1.55 (s, 6H), 2.36 (s, 3H), 4.34 (s, 2H), 4.92 (s, 2H).

6-Fluoro-α^4, 3-*O*-isopropylidenepyridox-5-yl thiocyanate ( ). To a solution of 514 mg (1.9 mmol) of 6-fluoro-α^4, 3-*O*-isopropylidenepyridox-5-yl bromide ( ) in 5 ml ethanol, was added 180 mg (2.2 mmol) NaSCN. The mixture was refluxed overnight. TLC (eluent: 10% EtOAc in hexane) confirmed complete conversion of the starting material. The solvent was evaporated *in vacuo* and the crude product was purified by flash chromatography (eluent: 10% EtOAc in hexane) to give 316 mg (63 %) of a white solid. ^1H NMR ($CDCl_3$) δ 1.57 (s, 6H), 2.38 (s, 3H), 4.06 (s, 2H), 4.97 (s, 2H). MS (m/z)

(6-Fluoro-α^4, 3-*O*-isopropylidenepyridox-5-yl) trifluoromethyl sulfide ( ). To 4.5 ml of a 0.5 M solution of Me_3SiCF_3 in THF (2.25 mmol) was added 300 mg (1.1 mmol) 6-fluoro-α^4, 3-*O*-isopropylidenepyridox-3-yl thiocyanate ( ) and 0.2 ml of a 1 M solution of TBAF in THF (Aldrich). The reaction mixture was stirred overnight. The solvent was evaporated *in vacuo* and the crude product was purified by flash chromatography (eluent: 10% EtOAc in hexane) to give

108 mg (31 %) of a colorless oil. ^{1}H NMR ($CDCl_3$) δ 1.55 (s, 6H), 2.35 (s, 3H), 3.90 (s, 2H), 4.91 (s, 2H). MS (m/z)

(6-Fluoropyridox-5-yl) trifluoromethyl sulfide ( ). To a solution of 108 mg (0.34 mmol) of (6-fluoro-α^4, 3-*O*-isopropylidenepyridox-5-yl) (trifluoromethyl) sulfide ( ) in 3 ml dioxane was added 1.5 ml 1 M HCl. The reaction mixture was stirred at 80 °C for 3 h and subsequently neutralized with aqueous K_2CO_3. The aqueous mixture was extracted 3 times with 10 ml EtOAc. The combined organic layers were dried on $MgSO_4$, filtered, and the solvent was evaporated *in vacuo* to give 80 mg (86 %) of a white solid.
^{1}H NMR (dmso-d_6) δ 2.31 (s, 3H), 4.33 (s, 2H), 4.72 (d, J = 4 Hz, 2H), 5.97 (d, 1H), 9.15 (br, 1H). MS (m/z)

6-Fluoro-α^4, 3-*O*-isopropylidenepyridox-5-yl azide ( ). To a solution of 780 mg (3 mmol) 6-fluoro-α^4, 3-*O*-isopropylidenepyridox-5-yl chloride (**8**) in 5 ml DMF was added 650 mg (10 mmol) NaN_3. The reaction mixture was stirred under an argon atmosphere for 16 h, allowed to cool to room temperature, and poured into 50 ml water. The aqueous layer was extracted three times with 50 ml EtOAC, and the combined organic layers were washed three times with 50 ml water. The organic layer was dried on $MgSO_4$, filtered, and the solvent was evaporated *in vacuo*. Flash chromatography (eluent: 10% EtOAc in hexane) gave 713 mg (90%) of a colorless oil. ^{1}H NMR ($CDCl_3$) δ 1.55 (s, 6H), 2.36 (s, 3H), 4.27 (s, 2H), 4.88 (s, 2H).

6-Fluoro-α^4, 3-*O*-isopropylidenepyridox-5-yl amine ( ). To a solution of 700 mg (2.7 mmol) of 6-fluoro-α^4, 3-*O*-isopropylidenepyridox-5-yl azide ( ) in 50 ml EtOH was added 400 mg 5% Pd on C. The reaction mixture was hydrogenolyzed at 20 psi for 16 h at room temperature. The reaction mixture was filtered over Celite and evaporation of the solvent in vacuo gave pure amine in an 83% yield in the form of a slowly solidifying oil. ^{1}H NMR ($CDCl_3$) δ 1.52 (s, 6H), 2.33 (s, 3H), 3.73 (s, 2H), 4.97 (s, 2H).

***N*-[6-Fluoro-α^4, 3-*O*-isopropylidenepyridox-5-yl]-2,2,2-trifluoroacetamide ( ).** To a solution of 250 mg (1.1 mmol) pyridoxyl amine ( ) in 5 ml methanol was added a solution of 180 μl SETFA (1.3 mmol) in 3 ml methanol. The reaction mixture was stirred overnight. TLC (eluent: 25 % EtOAc in hexane) indicated complete conversion of the starting material. The solvent was evaporated *in vacuo* and the crude product was purified by flash chromatography (eluent: 25 % EtOAc in hexane) to give 292 mg (81%) of a white solid. ^{1}H NMR ($CDCl_3$) δ 1.54 (s, 6H), 2.35 (s, 3H), 4.36 (d, J = 6.0 Hz, 2H), 5.03 (s, 2H), 6.8 (br, 1H).

***N*-[6-Fluoropyridox-5-yl]-2,2,2-trifluoroacetamide ( ).** To a solution of 104 mg (0.32 mmol) of *N*-[6-fluoro-α^4, 3-*O*-isopropylidenepyridox-5-yl]-2,2,2-trifluoroacetamide ( ) in 5 ml 1,4-dioxane, was added 1 ml 1M HCl. The clear solution was stirred for 4 h at 80 °C. After the reaction mixture was allowed to cool to room temperature, the pH was adjusted to 7 with 1 M NaOH. The solvents were evaporated *in vacuo* and the remaining white solid was triturated with 10 ml EtOAc and filtered. The filtrate was dried, filtered, and the solvent was evaporated *in vacuo* to leave 86 mg (100 %) of a white solid. ^{1}H NMR (dmso-d_6) δ 2.28 (s, 3H, major isomer), 4.41 (br, 2H), 4.68 (s, 2H, major isomer).

Acknowledgments
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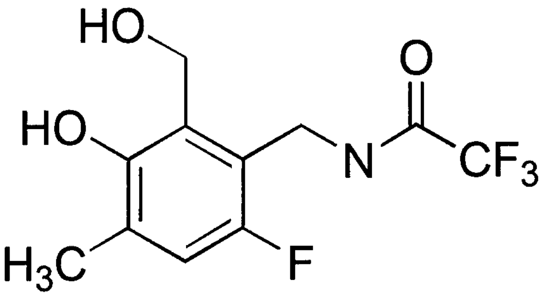
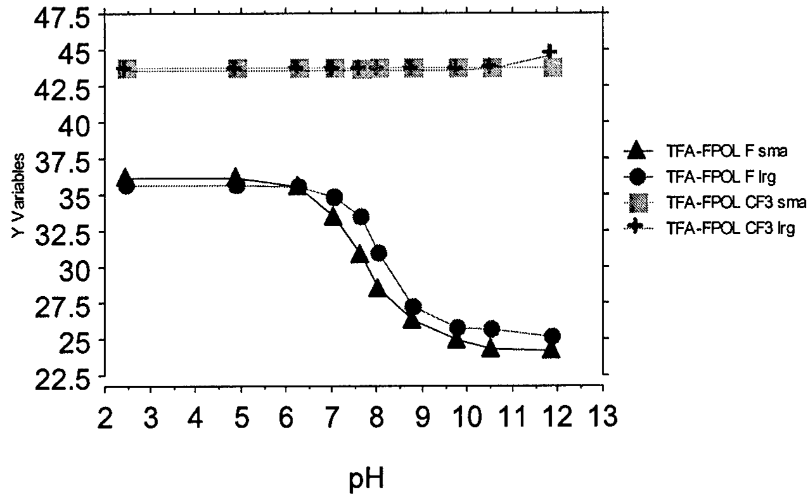
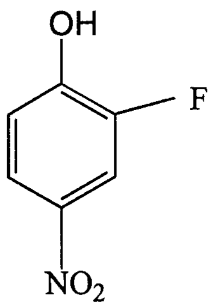
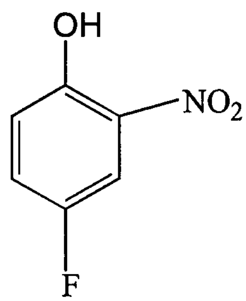
Appendix 6

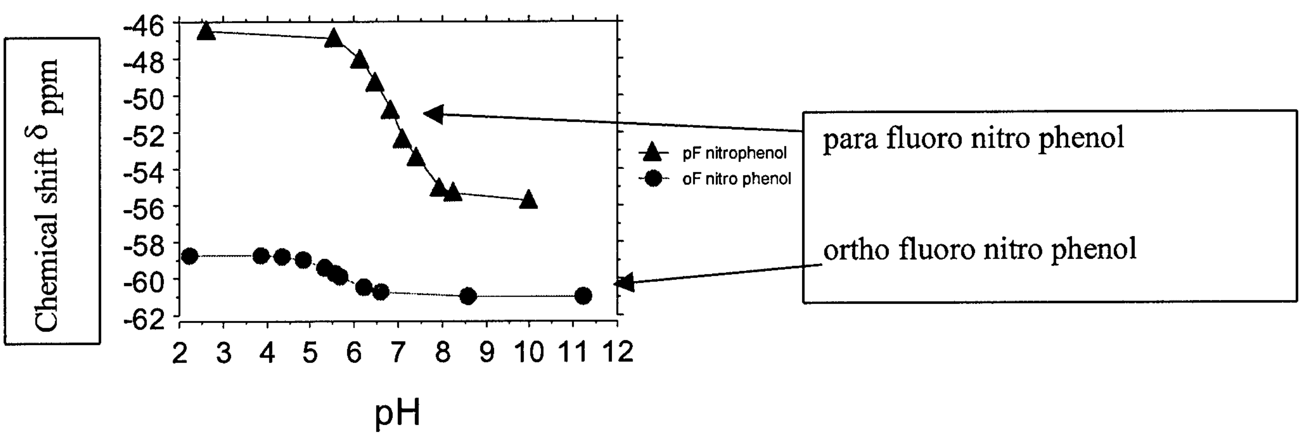




Y Variables
pH
TFA-FPOL F sma
TFA-FPOL F lrg
TFA-FPOL CF3 sma
TFA-FPOL CF3 lrg







Chemical shift δ ppm
pH
pF nitrophenol
oF nitro phenol
para fluoro nitro phenol
ortho fluoro nitro phenol